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Production of acetone–butanol–ethanol (ABE) in a continuous flow bioreactor using degermed corn and *Clostridium beijerinckii*

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Abstract

An examination of the sustainability of the long-term cultivation of *C. beijerinckii* BA101 in degermed corn/saccharified degermed corn based P2 medium has been described in this work. It was found that long-term continuous cultivation of *C. beijerinckii* BA101 in a degermed corn based medium was not possible due to the instability of the gelatinized degermed corn starch during storage often called ''retrogradation''. Using this substrate, continuous ABE fermentation was run for 228 h, before the fermentation turned acidogenic. However continuous fermentations of saccharified degermed corn with normal and half P2 medium nutrients were successful. In saccharified degermed corn continuous fermentation, ABE concentration up to 14.28 g/L was achieved at a dilution rate of 0.03 h^{-1}. This work demonstrated that byproduct (germ/oil, corn fiber) credit can be obtained by fermenting saccharified degermed corn in continuous flow bioreactors. Additionally significant savings can be achieved by supplementing with half of normal P2 medium nutrients.

Keywords: ABE fermentation; Degermed corn; Saccharified degermed corn; Retrogradation; *Clostridium beijerinckii* BA101

1. Introduction

A fermentation process that can be operated in a continuous mode may provide some advantages over a batch process. For example in a continuous flow reactor, only one batch of inoculum culture would be needed over the course of acetone–butanol–ethanol (ABE) production period, the volume of the fermentor can be reduced without a reduction in productivity, and the time necessary for cleaning and sterilization of the equipment would be drastically reduced. In addition, the continuous flow culture system is an important research tool for the determination of parameters responsible for the changes in the physiology and activity of solvent-producing clostridia [1]. The ABE fermentation process is of interest for chemical/fuel production from renewable resources. Butanol, a potential fuel extender is currently used as a feedstock chemical in the plastic industry and as a food grade extractant in the food and flavor industry. Currently, the annual butanol market in the United States is about 2.9 billion pounds.

Corn (composition—61% starch, 3.8% corn oil, 8.0% protein, 11.2% fiber and 16.0% moisture; yellow dent variety) can be used to produce ABE where starch is converted to the final product and the remaining components are left behind in the fermentation broth. Economic evaluations demonstrated that separation of germ/corn oil and corn fiber prior to fermentation results in improved economics of butanol production [2,3]. These byproducts (germ/corn oil and corn fiber) can be sold for byproduct credit.

In the early 1990s, our laboratory developed a hyper-amylolytic, hyper-butanol producing solventogenic *Clostridium* designated *C. beijerinckii* BA101 that is efficient in conversion of starch to ABE [4]. The genetic amplification of the extracellular amylases allows for efficient simultaneous saccharification and fermentation of starch-based biomass to butanol in batch fermentation [5]. Although a significant number of studies have been carried out on continuous ABE

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[1] Mention of trade names of commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

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production, most if not all, have used simple sugars as carbon sources [6–8]. However, the use of whole corn or degermed corn has not been investigated in continuous ABE fermentation to further strengthen the economics of ABE production from this substrate. Successful use of this substrate in combination with byproduct credit would make ABE fermentation economically more attractive.

Furthermore, batch studies on ABE production from degermed corn suggested that this substrate contains some of the nutrients that are essential for growth of *C. beijerinckii* BA101 and fermentation [9]. Hence, fermentation may not necessitate supplementation of all the nutrients required by the culture. With these views, the overall objective of this investigation was to examine the continuous fermentation of degermed corn to ABE using *C. beijerinckii* BA101. Since use of degermed corn as a fermentation substrate results in a significant byproduct credit, and continuous fermentation results in numerous advantages enumerated in this section, these studies were considered novel.

2. Materials and methods

2.1. Degermed corn liquefaction and saccharification processes

Corn was cleaned, tempered and channeled into the degerminator. The germs containing corn oil were removed for oil extraction, and the degermed corn was ground and sieved into appropriate particle size. The meal (350–400 g) was mixed with 0.05 M phosphate buffer (pH 6.9) to achieve a total volume of 1 L and sieved once using cheesecloth. This was called ''degermed corn'' and used for continuous fermentation. Starch gelatinizes when heated in the presence of excess water. Liquefaction and gelatinization of degermed corn starch were carried out in water bath at 100 °C and the starch was hydrolyzed by adding 2 mL (102 U/mL) thermostable α-amylase (Sigma, St. Louis, MO) enzyme to produce predominantly maltose and oligosaccharides. The slurry was liquefied until a 12 DE (Dextrose Equivalent) was achieved. DE was expressed as ''Amount of reducing sugars expressed as glucose (g)/total carbohydrate (g)) × 100''. The liquefied degermed corn was composed of 35–40% dry solids as reducing sugars, dextrin, and fiber. The breakdown of starch drastically reduced the viscosity of the gelatinized degermed cornstarch solution. The mash was cooled and the pH was adjusted to 4.5 with 1 M HCl and transferred into saccharification reactor, followed by raising the temperature to 45 °C. One milliliter (400 U/mL) of glucoamylase (Archer Daniels Midland Co., Decatur, IL) was added and saccharification of the liquefied starch to glucose was completed in 2–3 h.

2.2. Microorganism, culture maintenance and fermentation conditions

C. beijerinckii BA 101 was used for these studies. Laboratory stocks of *C. beijerinckii* BA101 were routinely maintained as spore suspension in sterile double distilled water (ddH_2O) at 4 °C. Spores (200 μL) were heat shocked for 10 min at 80 °C followed by cooling in the anaerobic chamber for 5 min. The culture was inoculated into 20 mL Tryptone–glucose–yeast extract (TGY) medium (in 50 mL screw capped Pyrex bottle) and was incubated anaerobically for 15–16 h at 35 ± 1 °C until an optical density of 1.0–1.2 was achieved at 600 nm (Beckman Du 640 Spectrophotometer).

2.3. Continuous cultivation of C. beijerinckii BA101

Continuous cultivation of *C. beijerinckii* BA101 was carried out in P2 medium containing 40–45 g/L degermed corn, saccharified degermed corn (55–60 g/L) or glucose (55–60 g/L) in a 2 L continuous flow bioreactor (New Brunswick Scientific Co., New Brunswick, NJ). The medium which contained 1 g/L yeast extract (Sigma Chemicals, St. Louis, MO) in addition to sugar or degermed corn was autoclaved at 121 °C for 15 min followed by cooling under oxygen free nitrogen gas environment which was created by sweeping the gas across medium surface. Upon cooling the medium, 10 mL of each of the stock solutions {buffer: KH_2PO_4 50 g/L, K_2HPO_4 50 g/L, ammonium acetate 220 g/L; Vitamins: *para*-amino-benzoic acid 0.1 g/L, thiamin 0.1 g/L, biotin 0.001 g/L; mineral: $MgSO_4{\cdot}7H_2O$ 20 g/L, $MnSO_4{\cdot}H_2O$ 1 g/L, $FeSO_4{\cdot}7H_2O$ 1 g/L, NaCl 1 g/L; [10]} were added to 920 mL of medium. It should be noted that the medium was prepared in such a way that the above sugar/substrate concentrations were obtained after addition of stock solutions.

One liter medium was prepared in the bioreactor as described above. The bioreactor was inoculated with 50 mL inoculum of highly motile cells of *C. beijerinckii* BA101. The fermentation was allowed to proceed in the batch mode for 24 h, after which fermentation feed medium was continuously pumped into the bioreactor at a dilution rate of 0.03 h^{-1}. The bioreactor was maintained at 35 ± 1 °C. The temperature of the feed medium was maintained at room temperature (21 °C). Fresh feed was prepared every 72 h. The fermentation could not be run with >45 g/L degermed corn feed solution due to the viscous nature of the feed. During the course of fermentation, 2 mL samples were collected at regular intervals and examined for acetone, butanol, ethanol, residual starch/sugar and acids. Unless otherwise stated, all continuous fermentations were carried out in duplicates and results reported are average of two fermentations.

2.4. Enzymatic hydrolysis of degermed corn starch

A mixture of 1.0 g [4.5% (w/v)] gelatinized degermed corn and 1.0 mL of 0.1 M phosphate buffer (pH 5.5) were equilibrated at 50 °C in a water bath for 5 min. Alpha-amylase (50 μL) (Sigma Chemicals, St. Louis, MO) solution was added and the sample was further incubated for 45 min and boiled for 5 min to stop the reaction. Reducing sugar was determined using the 3,5-dinitrosalicyclic acid (DNSA) method [11].

2.5. Acid hydrolysis of degermed corn

Hydrolysis was carried out by incubating 1.0 g (4.5%, w/v) degermed corn (from feed medium) or 228 h degermed corn sediment with 1 M HCl (9 mL) at 100 °C for 2 h. The sample was assayed immediately for reducing sugars [11]. The degree of hydrolysis (DH) [12] was calculated as reported elsewhere [13].

2.6. Analytical procedures

ABE and acids (acetic and butyric) were measured using a 6890 Hewlett-Packard Gas Chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector (FID) and 1.8 m × 2 mm glass column (10% CW-20 M, 0.01% H_3PO_4, support 80/100 Chromosorb WAW). Productivity for the continuous fermentation was calculated as total ABE concentration (g/L) multiplied by dilution rate (D/h). Fermentation time for continuous fermentation was defined as the total running time. Being a biological system, fluctuations in ABE concentration is normal and was of the order of 6–11%.

Glucose concentration was determined using a hexokinase and glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO) coupled enzymatic assay as described previously [14]. In order to measure glucose/reducing sugars, the fermentation broth was centrifuged using spectrafuge 16 M centrifuge (Labnet International Inc. Woodbridge, New Jersey) at 16,000 × *g* for 3 min at 4 °C. Ten microliters of standard solution was mixed with glucose (HK 20) reagent (1.0 mL) and incubated at room temperature for 5 min. A blank (deionized water) (10 μL) was incubated with the reagent and was used for zero adjustment of the spectrophotometer.

Starch concentration of the samples was determined using a modified method of Holm et al. [15] as described previously [13]. The amount of sample that was taken for these studies was 350 mg rather than 250 mg. The concentration of starch (%) in degermed corn was calculated as: [mg glucose × 25^a × 50^a × 0.9^b × 100]/[sample weight (350 mg)], where *a* = dilution factor; *b* = correction glucose to glucan.

3. Results

3.1. Continuous production of ABE from glucose

In order to evaluate the performance of degermed corn continuous fermentation, a control experiment was run in which glucose was used as the substrate. The experiment was run for 504 h and the results of ABE production are shown in Fig. 1A. In this system an average ABE concentration of 9.32 g/L was produced at a dilution rate of 0.03 h^{-1} thus resulting in a productivity of 0.28 g/L/h. During the 504 h of operation, there was no pH control and no sign of degeneration of the culture. The average concentrations of the individual components were acetone 2.67, butanol 6.46, and ethanol 0.20 g/L. The average concentrations of acetic, butyric, and total acids were 2.10, 0.38, and 2.48 g/L, respectively (Fig. 1B).

3.2. Continuous production of ABE from degermed corn

Next, an experiment was run with degermed corn as feed (Fig. 2A and B). In this experiment a maximum concentration of 5.89 g/L of butanol was achieved at 60 h. At this stage, the total concentrations of ABE and starch in the effluent were 8.98, and 8.1 g/L, respectively. Further continuation of fermentation resulted in a gradual decrease of ABE production and an increase in acids production. At 228 h, concentrations of acetone, butanol, ethanol, acetic acid, and butyric acid were 0.41, 1.31, 0.10, 2.31, and 1.49 g/L, respectively. At this point, the culture turned acidogenic with total concentrations of acids of 7.17 g/L and starch 26.2 g/L. Hence, fermentation was stopped. It was observed that a significant amount of sediment had accumulated in the bottom of the reactor (up to 8.6 cm high; bioreactor vessel height, 21.5 cm).

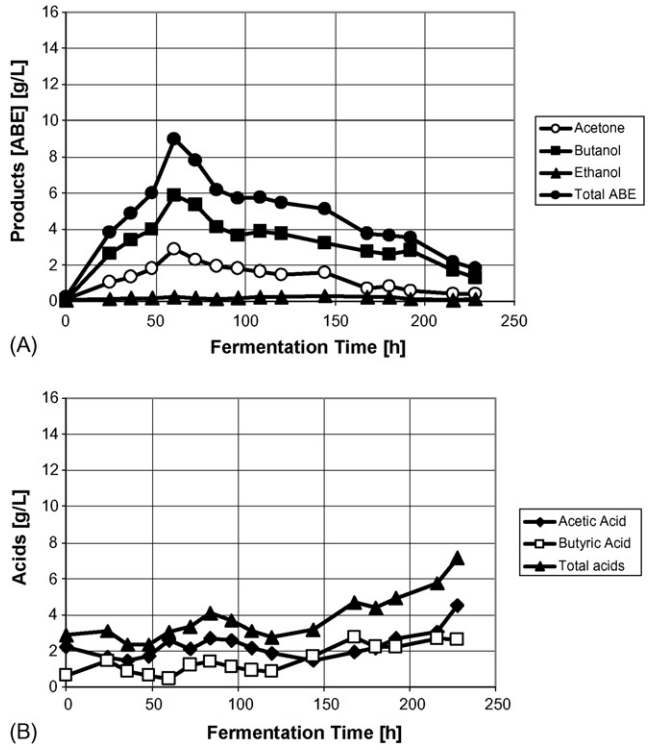

Fig. 2. Continuous production of ABE from degermed corn using *C. beijerinckii* BA101: (A) ABE vs. fermentation time and (B) acids vs. fermentation time.

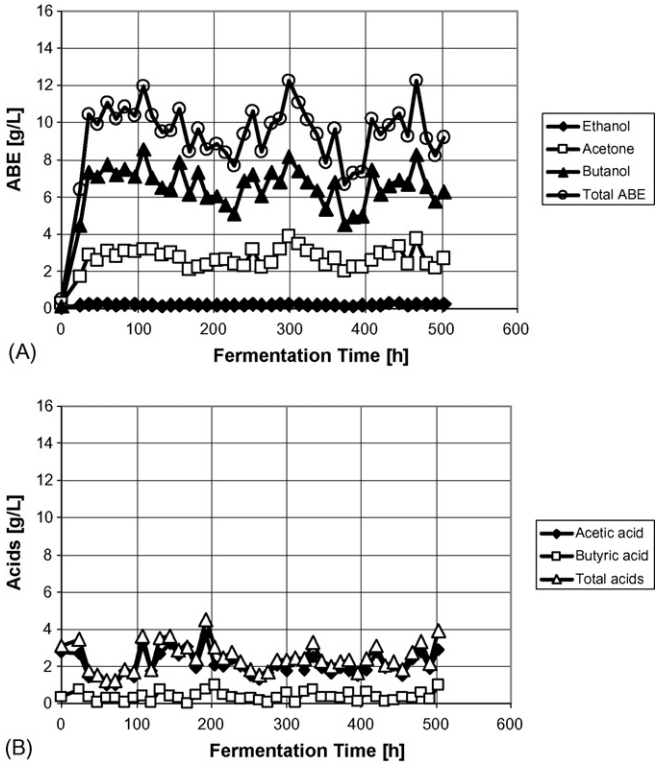

Fig. 1. Continuous production of ABE from glucose using *C. beijerinckii* BA101: (A) ABE vs. fermentation time and (B) acids vs. fermentation time.

In the above experiment, it was investigated whether *C. beijerinckii* BA101 hydrolyzed degermed corn to glucose faster than it utilized the latter for cell growth and ABE production. For this, we analyzed the effluent for presence of glucose. In the effluent, a maximum residual glucose level of 0.15 g/L was observed at 120 h. Beyond 120 h of fermentation, the glucose level in the effluent was $\ll$0.15 g/L. Also, the degree of hydrolysis of degermed corn at various time periods was measured (Fig. 3). The degree of hydrolysis of degermed corn was 72.3% at zero time and 49.5% at 72 h. The degree of

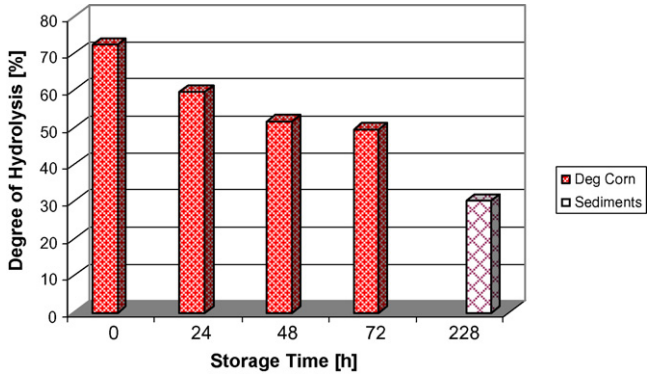

Fig. 3. Effect of storage on the degree of hydrolysis of gelatinized degermed corn. One hundred percentage degree of hydrolysis = 16.26 g/L reducing sugars.

hydrolysis of sediments that were removed from the continuous flow bioreactor (228 h) was found to be 30.2% (Fig. 3).

3.3. Continuous production of ABE from saccharified degermed corn containing full P2 nutrients

Since degermed corn fermentation became acidogenic and terminated prematurely, we investigated the use of saccharified degermed corn for continuous fermentation. The results of this successful fermentation (dilution rate 0.03 h^{-1}) are shown in Fig. 4A and B. The fermentation was run for 504 h as opposed to degermed corn fermentation for 228 h. After 504 h of continuous operation the fermentation was stopped intentionally. In this fermentation, ABE concentration up to 14.28 g/L was achieved. An average concentration of ABE that was achieved in this run was 9.70 g/L (acetone 3.08 g/L, butanol 6.33 g/L, and ethanol 0.28 g/L) over a period of 504 h. The concentrations of acetic and butyric acids were 2.49, and 0.72 g/L, respectively. In this fermentation ABE productivity of 0.29 g/L/h was achieved. There was no significant difference between this run and the control.

3.4. Continuous production of ABE from saccharified degermed corn containing half of P2 nutrients

In order to investigate the effect of P2 medium on saccharified degermed corn, a continuous fermentation was run with this substrate and half of normal P2 medium supplementation. The fermentation was run under the same conditions. In this fermentation, total ABE concentration up to 14.16 g/L was obtained and the fermentation was run for 504 h (Fig. 5A and B). The average concentration of ABE was 10.12 g/L resulting in a productivity of 0.30 g/L/h. The average concentrations of the individual components (ABE, acetic and butyric acid) were 3.11, 6.78, 0.26, 2.67, and 0.75 g/L, respectively. After 504 h of operation the experiment was stopped intentionally. A continuous ABE fermentation involving saccharified degermed corn without P2 supplementation was not successful (data not shown).

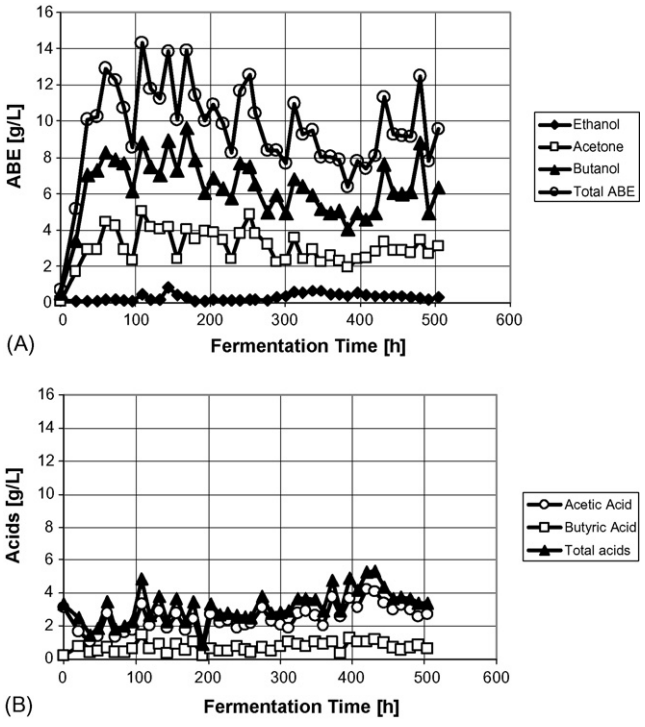

Fig. 4. Continuous production of ABE from saccharified degermed corn using *C. beijerinckii* BA101: (A) ABE vs. fermentation time and (B) acids vs. fermentation time.

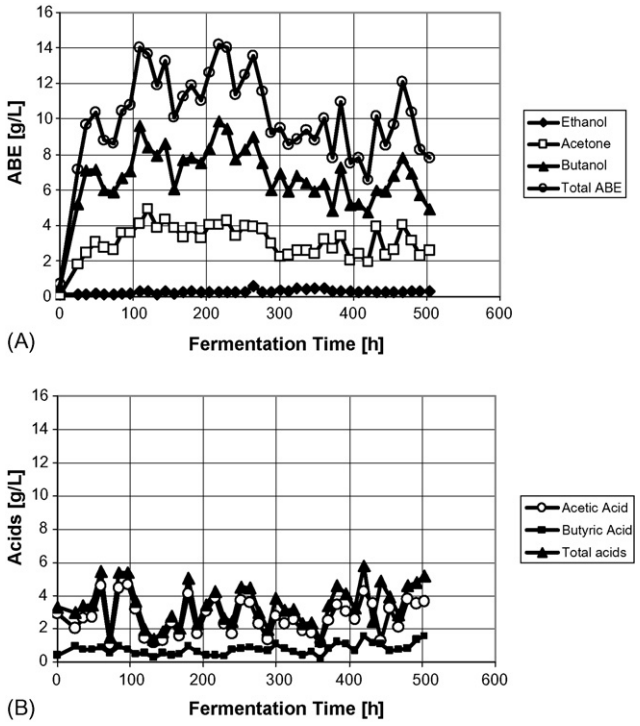

Fig. 5. Continuous production of ABE from saccharified degermed corn using *C. beijerinckii* BA101 and half of P2 nutrients: (A) ABE vs. fermentation time and (B) acids vs. fermentation time.

4. Discussion

In the degermed corn fermentation, the ability of the culture to produce ABE started to decline after a period of 60 h, and after 168 h of fermentation the culture became acidogenic. There were three possibilities of getting the fermentation acidogenic: (i) presence of an excess amount of nutrients in the degermed corn feed medium as it also contained P2 medium nutrients; (ii) inefficient utilization of degermed corn starch; (iii) slow secretion of amylolytic enzymes by the fermenting microorganism in the bioreactor. Slow secretion of amylolytic enzymes was disregarded as the culture used starch efficiently in batch reactors [5,9]. A comparison of saccharified degermed corn fermentation with degermed corn fermentation (both supplemented with normal P2 medium nutrients) suggested that the degermed corn fermentation did not become acidogenic due to the presence of an excess amount of nutrients.

In the degermed corn fermentation effluent, the residual glucose level was $\ll$0.15 g/L. It has been reported that as the concentration of substrate falls below the threshold concentration required to sustain ABE production in the reactor [16–19], the

rate of acid reassimilation by *C. beijerinckii*/*C. acetobutylicum* decreases, thus resulting in accumulation of acids in the bioreactor. In ABE fermentation, acids are metabolic intermediates and are produced before they are reassimilated for acetone and butanol production [14]. Availability of excess sugar in the bioreactor is essential for both the onset (assimilation of acids) and the maintenance of solvent (ABE) production [20]. This suggested that the culture was not able to hydrolyze degermed corn starch efficiently, possibly due to the deterioration (retrogradation) of the gelatinized degermed corn starch feed.

In a process commonly known as gelatinization, starch granules are disrupted by heating in an excess of water, which renders the molecules fully accessible to hydrolytic enzymes [21]. However, gelatinized starch gels are thermodynamically unstable structures, and on cooling re-association of the starch molecules through H-bonding (involving both amylose and amylopectin) occurs with a corresponding increase in viscosity, a phenomenon termed as ''retrogradation''. The mechanism of retrogradation has been described elsewhere [22,23]. Retrogradation has been shown to significantly lower the enzymatic susceptibility of gelatinized starch to hydrolysis [12,24,25]. The physical and chemical properties of gelatinized starch solutions can change during storage due to retrogradation [23,26,27].

Retrogradation can also be called as ''increased resistance to amylolysis'' and involves both amylose and amylopectin components of starch [12,21,28]. While the process of amylose retrogradation sets in moments after cooling the gelatinized starch, the amylopectin retrogradation begins several days later. The effect of storage on the susceptibility of gelatinized degermed corn starch indicated that the degree of hydrolysis of gelatinized degermed corn starch decreased (72.3–49.5%) within short period of time (0–72 h) (Fig. 3) suggesting that the decrease in the degree of hydrolysis was as a result of amylose retrogradation. In addition, the degree of hydrolysis of the degermed corn sediments retrieved from the bioreactor after the termination of fermentation (228 h) revealed a further decline in degree of hydrolysis (30.2%) suggesting that amylopectin retrogradation may have also taken place and therefore contributed to further lower the degree of hydrolysis of the degermed corn sediments. This explanation and the results presented in Fig. 3 confirmed that degermed corn starch retrograded, thus affecting the degermed corn continuous fermentation. This observation is in accordance with pure starch retrogradation as we investigated earlier [13]. A practical way of eliminating the problems associated with starch retrogradation is by saccharification of degermed corn before use in the continuous production of ABE. It should be noted that the saccharified degermed corn contains 55–60 g/L glucose. Continuous cultivation of *C. beijerinckii* BA101 in saccharified degermed corn medium resulted in solventogenic process of fermentation as demonstrated by the lower total acids maintained in the bioreactor until the fermentation was intentionally terminated after 504 h of fermentation (Fig. 4B and 5B). The fermentation was successful due to the elimination of degermed corn starch retrogradation reactions and availability of sufficient amounts of sugars (saccharified degermed corn) in the bioreactor throughout the fementation period.

In continuous ABE fermentation, fluctuations in ABE concentration (Figs. 1,2 and 4,5) were recorded in the effluent throughout the fermentation, which is typical of ABE continuous fermentation systems [6–8]. In continuous ABE fermentation, as the solventogenic clostridia cells continue to undergo shifts between acidogenesis and solventogenesis, the bioreactor gets composed of a mixture of actively dividing cells (acidogenic), non-dividing cells (solventogenic), sporulated cells, and dead cells. The oscillatory behavior of the ABE concentration in the bioreactor and effluent during the continuous ABE fermentation by *C. beijerinckii* BA101 indicates that there are periods of time when the non-dividing cells (solventogenic) concentration is comparatively high or low in the bioreactor resulting in high or low ABE production. Culture degeneration is a feature usually associated with genetic change and takes place over a period of time, particularly during continuous fermentation. In our study, there was no degeneration and the culture was stable for 504 h after which the process was intentionally terminated (Figs. 1,2 and 4,5).

A comparison of the experiment where half of P2 medium nutrients was used with the saccharified degermed corn and saccharified degermed corn with normal P2 medium nutrients suggested that fermentation of saccharified degermed corn does not require supplementation with normal P2 medium. This was as a result of presence of nutrients in the saccharified degermed corn. Reduction in nutrient supplementation would result in an economic production of ABE from saccharified degermed corn. However, a continuous ABE fermentation involving saccharified degermed corn without P2 supplementation was not successful due to lack of some nutrients essential for the growth and maintenance of *C. beijerinckii* BA101 cells.

5. Conclusions

Examination of the sustainability of the long-term cultivation of *C. beijerinckii* BA101 in degermed corn/saccharified degermed corn based P2 medium has been described in this work. It was found that long-term continuous cultivation of *C. beijerinckii* BA101 in a degermed corn based medium was not possible due to the instability of the gelatinized degermed corn starch during storage often called retrogradation. Saccharified degermed corn, which is not prone to retrogradation sustained long-term cultivation of *C. beijerinckii* BA101 during continuous ABE production. Therefore, continuous fermentation of degermed corn for ABE production can be sustained for a longer period of time if the degermed corn is saccharified before use as feed medium. There was no significant difference between continuous fermentations of saccharified degermed corn supplemented with full P2 medium nutrients and that supplemented with half P2 medium nutrients. Hence this work demonstrated that byproduct (germ/oil, corn fiber) credit can be obtained by fermenting saccharified degermed corn in a continuous flow culture. Additionally, significant savings can be achieved by supplementing with half of P2 medium nutrients.

Acknowledgements

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